Xyrex® Prawnfresh™ Antibacterial Activity Assessment

A Scientific Report by

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Background

Global crustacean aquaculture is dominated by penaeid (shrimp/prawn) farming, the annual production of which far exceeds that of wild capture fisheries, with over 70% of the shrimp produced globally being sourced from aquaculture. Consequently, the commercial culture of penaeid shrimp is an extremely large global industry, worth many billions of dollars annually. The Pacific white shrimp, *Litopenaeus vannamei* is currently the most commercially-cultured crustacean species worldwide. It surpassed the Giant tiger prawn, *Penaeus monodon*, as the primary species of shrimp aquaculture in 2004, due mainly to the difficulty of breeding and the greater susceptibility to disease of the latter (http://fao.org/2009). Global aquaculture production of *L. vannamei* in 2007 was estimated as *ca*. 2,300,000 tonnes (http://fao.org/2009). This investigation will determine, using *in vitro* methods, the potential of and degree to which the Xyrex® products Prawnfresh™ and Prawnfresh+™ inhibit the growth of *Vibrio harveyi* (NCIMB 1280) and *Vibrio campbellii* (NCIMB 1894). Both of these bacterial species are known causative agents of luminescent Vibriosis, widely regarded to be the most significant bacterial disease affecting cultured juvenile and adult *L*. *vannamei* (Jayasree *et al*., 2006; Soto-Rodríguez *et al*., 2006).

Project aims

- To quantify the effectiveness of the Xyrex® products Prawnfresh™ and Prawnfresh+™ in interfering with or inhibiting the growth of the penaeid bacterial pathogens *Vibrio harveyi* (NCIMB 1280) and *Vibrio campbellii* (NCIMB 1894), and thereby their inferred activity against *Vibrio* spp. in general.

- Secondly, to ascertain whether dosage (concentration) and culture temperature are significant variables influencing any antibacterial activity of Prawnfresh™ and Prawnfresh+™.

Project deliverables

A scientific report provided to Xyrex Ltd. that describes the results obtained and draws conclusions about:

- The effectiveness of Xyrex® products Prawnfresh™ and Prawnfresh+™ in interfering with or inhibiting the growth of penaeid bacterial pathogens *Vibrio harveyi* (NCIMB 1280) and *Vibrio campbellii* (NCIMB 1894), and thereby their inferred activity against *Vibrio* spp. in general.
- The most effective dosages (concentrations) of Prawnfresh™ and Prawnfresh+™ and culture temperature for suppressing bacterial activity.

Materials and Methods

An antibacterial disc assay utilising the combined and modified methodologies of Bauer *et al.* (1966) and Pilet *et al.* (1995) was used to determine the effectiveness of Prawnfresh™ and Prawnfresh+™ in inhibiting the growth of *V. harveyi* (NCIMB 1280) and *V. campbellii* (NCIMB 1894). Twenty five millilitres of *V. harveyi* culture, previously grown up in TSB broth (plus 2% NaCl) for 18 h at 25^{o}C, was aseptically mixed with 325 ml of molten TSA (plus 2% NaCl) at 40^{o}C. Sufficient TSA powder and NaCl was used for a 350 ml final volume. The resulting agar-pathogen suspension was then aseptically dispensed into twenty 90 mm Petri dishes. This process was then repeated for *V. campbellii*.

Three concentrations of Prawnfresh™ and Prawnfresh+™ solutions, at 1, 2 and 4 parts per thousand (ppt), were tested using standard disc antibiotic susceptibility assay methods, as originally defined by Bauer *et al.* (1966). Test solutions were prepared as per the manufacturer's instructions and tested alongside a negative control (sterile 3.2% NaCl solution).

Autoclaved, 13 mm diameter discs of Whatman No. 17 antibiotic assay chromatography paper were prepared by pipetting 100 µl of test solution onto their surface, before being left to rest for 10 min to allow the solution time to infuse. Forty five discs were prepared for each solution. Four discs (representing the three test solutions plus the control) were positioned equidistantly, using flamed forceps, on each pathogen/agar plate. Five replicates plates (of each pathogen) were incubated at one of three temperatures (20^{o}C, 25^{o}C or 30^{o}C) to investigate the ability of Prawnfresh™ and Prawnfresh+™ to inhibit pathogen growth over a range of transport/processing conditions.

Each plate was then examined for evidence of growth interference/inhibition after 24 h and 48 h incubation. Such interference/inhibition is typically characterised by a region of reduced colony growth (clear agar) around the test disc. Any zone, or 'halo', of such pathogen growth interference or inhibition was subsequently recorded using digital

callipers (zone diameter in mm, including the 13 mm disc). The effective zone radius was then determined by subtracting the disc diameter (thirteen) and halving the subsequent value.

Results & Discussion

Only the results for the Prawnfresh™ test solutions are displayed as at no time did Prawnfresh+™ exhibit any detectable antagonistic or antibacterial activity against either *V. harveyi* (NCIMB 1280) or *V. campbellii* (NCIMB 1894).

It should be noted that the term growth interference will be used in preference to growth inhibition as the former is more accurate in describing the observed effects of Prawnfresh™ on the pathogens. The presence of bacterial growth (i.e. colonies) within the zone/halo surrounding the majority of discs precludes the effect being described as inhibition. These colonies, however, appeared reduced in terms of both their number and size in comparison with the area surrounding the control disc, although the observed effect was considerably less in the case of *V. campbellii* (Figures 2 & 3).

Although no measurements were taken, surviving plates (i.e. those not exhibiting excessive desiccation) were examined also at 96 h post inoculation. As at 24 and 48 hours the Prawnfresh+™ plates continued to exhibit no antagonistic activity against either pathogen. With regard to the Prawnfresh™ test plates, all *V. campbellii* inoculated plates were fully overgrown (not shown), i.e. by 96h the pathogen had overcome the antagonistic effects of the product. Halos of growth interference were still apparent on all *V. harveyi* inoculated plates for all temperatures and concentrations of the product; however, the zones surrounding the 1ppt treatment discs did appear to have diminished slightly, both in their size and effectiveness (Figures 2ii & 2iii).

A clear positive correlation is apparent between the concentration of the Prawnfresh™ solution used and the radial width of the halo of antagonism (zone of growth interference) (Figure 1). Thus the most effective dosage tested for interfering with the growth of both pathogens, over a 20-30^{o}C temperature range, was the 4ppt solution (Figure 1). Furthermore, standard deviation values indicate that incubation temperature does not appear to be a significant variable in the effectiveness of Prawnfresh™. Also in the case of *V. harveyi* no significant variation in zone width was apparent between the 24 and 48 hour values at both 25^{o}C and 30^{o}C (Figure 1). In the 96 hour observations the *V. harveyi* colonies present within the zones showed a slight increase in size; however, no variation was apparent in zone width and most significantly no new colonies were detectable (Figure 2iii).

The effectiveness of Prawnfresh™ in interfering with the growth of *V. campbellii* was markedly less than that observed for *V. harveyi* (Figure 1). Unlike *V. harveyi*, *V. campbellii* appeared to largely overcome the antagonistic effects of Prawnfresh™ at the lower concentrations of 1 & 2ppt within 48 hours, and within 96 hours for the 4ppt treatment (Figure 1). It is not unusual to observe variation in the effectiveness of antimicrobials between bacterial

strains and unless complete resistance is observed this could likely be overcome through the utilisation of an increased dose. However, this approach is not recommended when dealing with live animals, as excessive use may result in the creation of strains resistance to the agent's effects, as is the case with the prophylactic use of antibiotics in aquaculture (Li *et al*., 1999; Laganà *et al*., 2011). However, in the present case the agent in question, Prawnfresh™, is designed for use on harvested and therefore dead shrimp destined for human consumption. Consequently the likelihood of producing such resistant bacteria, and for them to become established in the wider microbial flora, is remote.

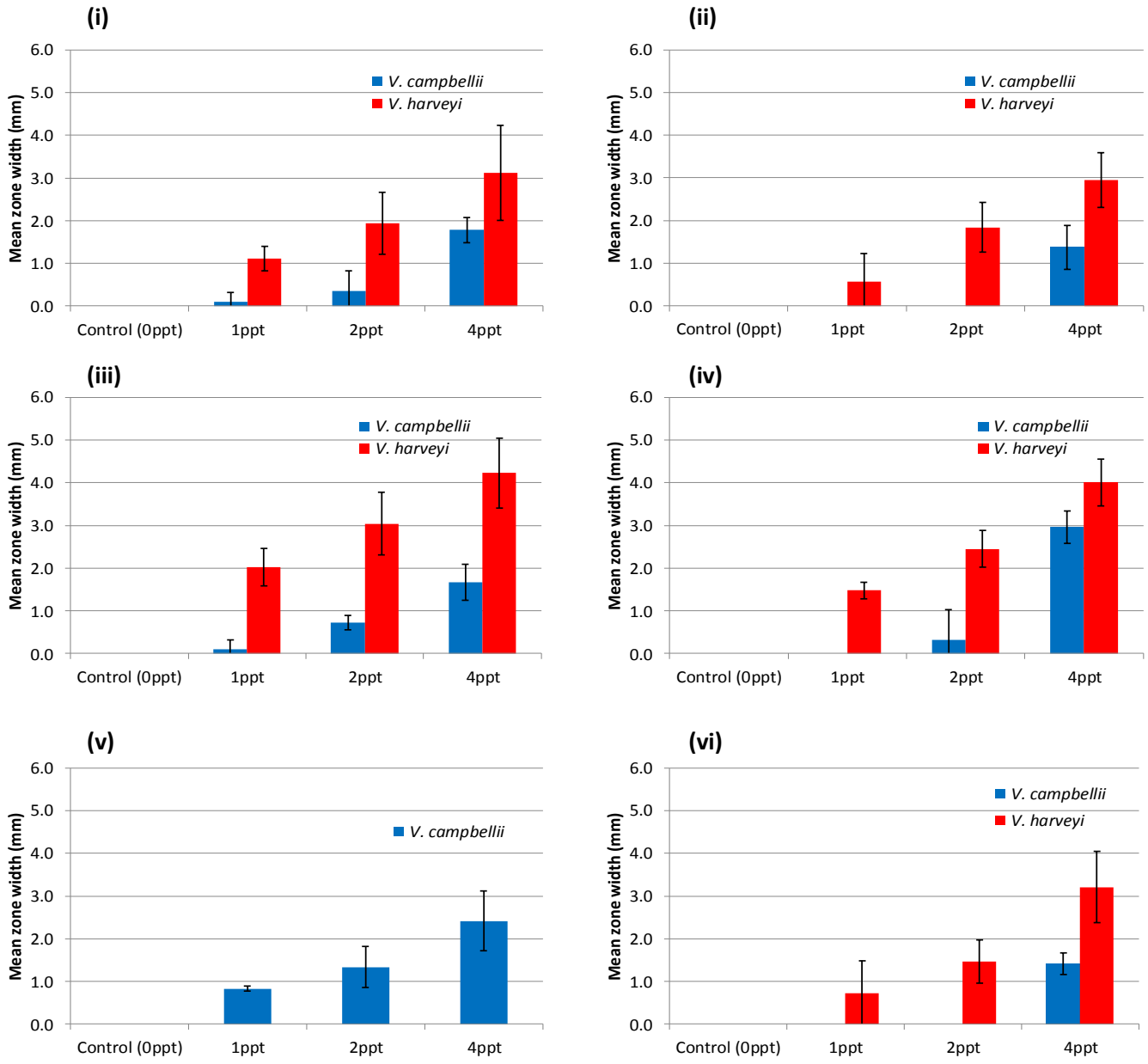

Figure 1. Mean radial width, in mm, of the growth interference zone/halo surrounding discs infused with solutions of 0, 1, 2 & 4 parts per thousand of Prawnfresh™, on *Vibrio harveyi* and *Vibrio campbellii* plates after **(i)** 24 hours at 30°C, **(ii)** 48 hours at 30 °C, **(iii)** 24 hours at 25 °C, **(iv)** 48 hours at 25 °C, **(v)** 24 hours at 20 °C and **(vi)** 48 hours at 20 °C. Data displayed as mean values +/- S.D. N=5 in each case.

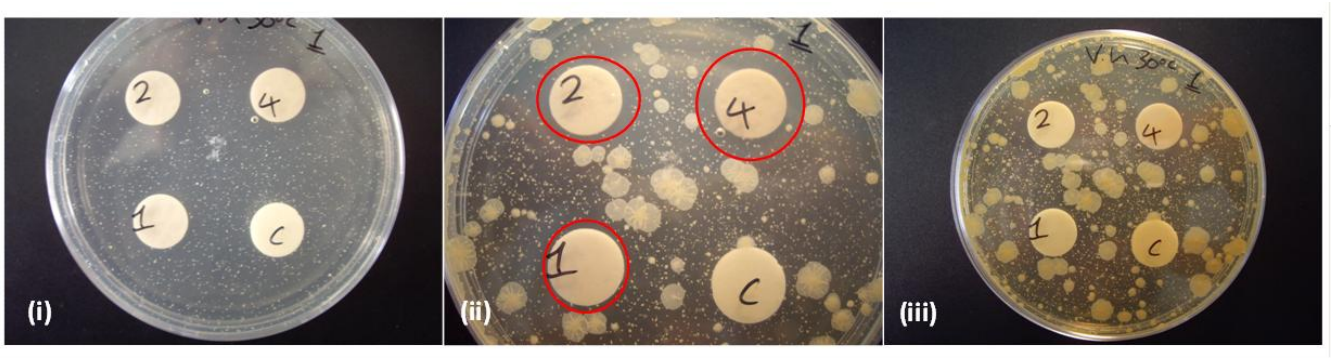

Figure 2. *V. harveyi* inoculated replicate plate 1 incubated at 30°C; **(i)** after 24 hours, **(ii)** after 48 hours with zones/halos of interference circled & **(iii)** after 96 hours. ('C' denotes control solution (3.2% NaCl); '1' denotes 1ppt Prawnfresh™ solution; '2' denotes 2ppt Prawnfresh™ solution and '4' denotes 4ppt Prawnfresh™ solution).

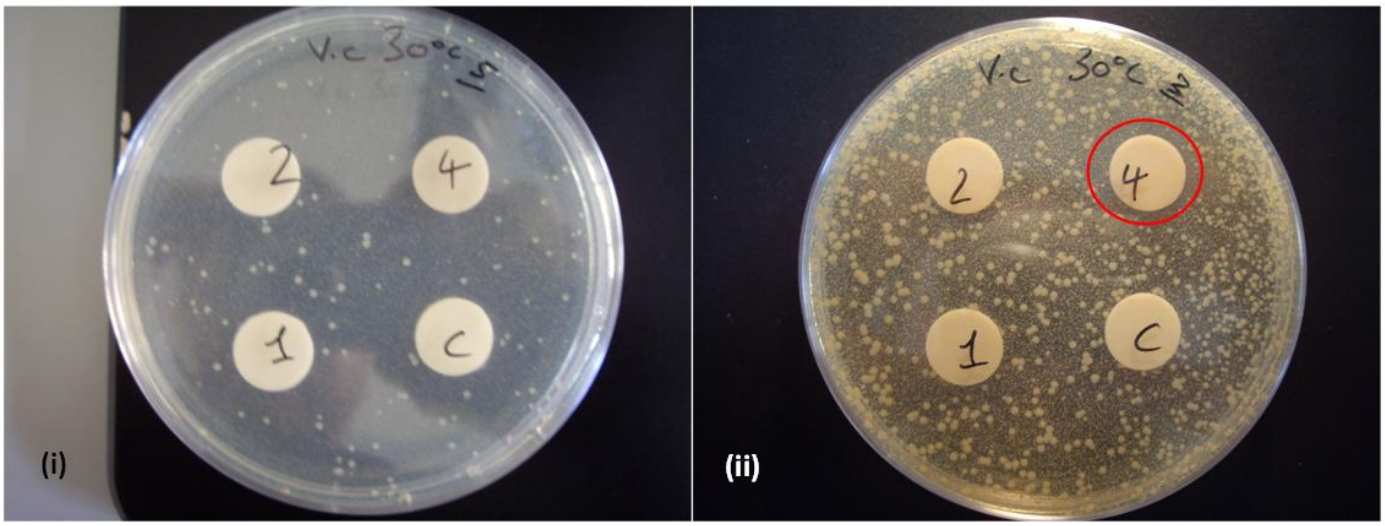

Figure 3. *V. campbellii* inoculated plates incubated at 30°C; **(i)** after 24 hours & **(ii)** after 48 hours with zone/halo of interference circled. ('C' denotes control solution (3.2% NaCl); '1' denotes 1ppt Prawnfresh™ solution; '2' denotes 2ppt Prawnfresh™ solution and '4' denotes 4ppt Prawnfresh™ solution).

Conclusions

- Prawnfresh™ displays the capacity to retard/interfere with the growth of both bacterial pathogens tested, *V. campbellii and V. harveyii*, but was significantly more effective in the case of *V. harveyi*.
- The greater the concentration of the Prawnfresh™ solution used the more effective it was in retarding/interfering with growth of *V. harveyi* (NCIMB 1280) and *V. campbellii* (NCIMB 1894). This will also most likely be the case for related species of *Vibrio* bacteria.
- Temperature, and also in the case of *V. harveyi* length of incubation period, do not appear to be significant variables in the effectiveness of Prawnfresh™ in interfering with the growth of the pathogens.
- The finding of a complete lack of any antagonistic activity by the test solutions of Prawnfresh+™ strongly indicates that components that are present in Prawnfresh™ but absent in Prawnfresh+™ are responsible for the antibacterial effects observed.
- Further to the above, the antagonistic effects on *Vibrio* species by Prawnfresh™ would likely be enhanced by increasing the concentration of these components. This would have a secondary benefit in that the overall recommended dosage of Prawnfresh™ (1ppt) could remain unaltered while providing increased bacterial antagonism.

Recommendations

- Further *in vitro* assessment to definitively isolate the active ingredient/ingredients responsible for the *Vibrio* growth interference observed.
- *In vivo* testing, to confirm the functional capacity of Prawnfresh™ to interfere with bacterial and particularly *Vibrio* growth. This would involve the assessment of the external colony forming units present on recently harvested shrimp (treated versus untreated).
- Although not the principal function of Prawnfresh™ its ability to interfere with the growth of two of the primary causative agents of luminescent Vibriosis could provide a useful additional marketing tool in demonstrating its superiority over other products in the marketplace, such as sodium metabisulphite.

Acknowledgements

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